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TITLE: Role of NF-Kappa B Signaling in X-Box Binding Protein 1 (XBP1)-Mediated Antiestrogen Resistance in Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. Rong Hu

CONTRACTING ORGANIZATION: Georgetown University
                                                 Washington, DC 20057-0001

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Role of NF-Kappa B Signaling in X-Box Binding Protein 1 (XBP1)-Mediated Antiestrogen Resistance in Breast Cancer

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
Most breast cancer patients who undertake antiestrogen therapy eventually suffers from antiestrogen resistance. Understanding its molecular mechanism is essential for identifying potential targets to overcome antiestrogen resistance. XBP1-S, an important regulator of the unfolded protein response (UPR), is found highly expressed in antiestrogen resistant breast cancer cells and tissues. XBP1-S is believed to function as an important antiestrogen resistance mediator as overexpression of XBP1-S is sufficient to drive resistancy to antiestrogens in MCF7 cells. In this study, we aim to investigate the mechanism of XBP1-mediated antiestorgen resistance, specifically the involvement of NFkappaB signaling. We found that XBP1 regulates NFkappaB signaling at least at two levels. One, XBP1-S regulates RelA expression at the mRNA level; Second, XBP1 regulates NFkappaB transcriptional activity through ERalpha signaling. Furthermore, inhibition of NFkappaB with either Parthenolide (small molecule inhibitor) or p65/RelA knockdown inhibits cell growth and antiestrogen resistance in XBP1-S overexpressed MCF7 cells. Taken together, NFkappaB signaling is required for XBP1-S mediated antiestrogen resistance. Future experiments will be performed in nude mice model to test the role of XBP1-NFkappaB signaling in antiestrogen resistance *in vivo*.

15. SUBJECT TERMS
X-Box Binding Protein-1, NF-kappa B, antiestrogen resistance, Breast cancer

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Table of Contents

I. Introduction:

Antiestrogen resistance is a major hurdle for endocrine therapy for ER+ breast cancer patients (1). The UPR major component XBP1 is a transcription factor that was shown to be up-regulated in antiestrogen resistant breast cancer cells and tumors (2-4). Overexpression of XBP1 is sufficient to promote resistance to antiestrogens in breast cancer (2). However, the underlying mechanisms remain to be clarified. NFkappaB signaling is known to be up-regulated in antiestrogen resistant cells and inhibition of NFkappaB resensitizes cells to antiestrogen (5). We hypothesize that NFkappaB is downstream of XBP1 signaling and mediates the antiestrogen resistance signaling.

II. Research Accomplishment Body:

Aim 1: Determine whether the XBP1-mediated antiestrogen resistance is mediated through NFkappaB signaling.

SubAim 1: Determine whether XBP1 regulates NFkappaB transcriptional activity.

We determined whether overexpression of XBP1 is sufficient to promote NFkappaB activity by overexpressing XBP1 in antiestrogen sensitive cells. To understand which isoform of XBP1 regulates NFkappaB activity, I proposed to overexpress XBP1(U) and XBP1(S) individually by also including a XBP1 nonspliceable mutant (nonS). However, we are not able to detect exogenous XBP1(U) unless the cells are treated with proteasomal inhibitor MG132. It appears that XBP1(U) is degraded too quickly in MCF7 cells for it to be detected by western blot -even when overexpressed under the CMV promoter (6). To ensure we have sufficient exogenous XBP1(U) to determine its function, we mutated the Lysines that are important for XBP1(U) ubiquitination and degradation (XBP1-K2R). The mutations were designed to alter the coding sequence of XBP1(U) only and remain to produce wild-type XBP1(S) after splicing (change the RNA splice site sequence but not the subsequently encoded amino acid sequence). Enhanced NFkappaB activity is observed in XBP1(S) and/or XBP1(U) overexpressed cells, suggesting both isoforms of XBP1 could regulates NFkappaB signaling (**Figure 1**).
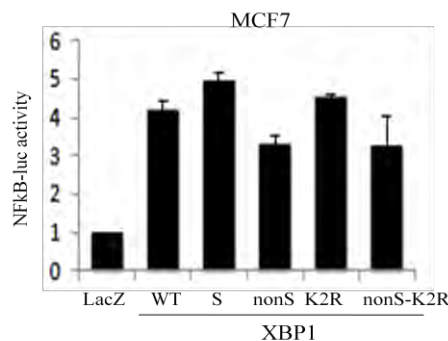

Figure 1. XBP1 regulates NFKappaB transcriptional activity. MC7 cells were co-transfected with NFkappaB-luciferase and XBP1 encoding constructs. NFkappaB transcriptional activity was measured by luciferase assay.

SubAim 2: Determine how XBP1 regulates RelA.

We measured RelA level in XBP1 overexpressing MCF7 cells. We observed that RelA is up-regulated when XBP1(S), but not XBP1(U) is overexpressed (**Figure 2)**. This result suggests that XBP1(S) controls RelA protein expression level in breast cancer cells. In future experiments, I will examine RelA mRNA level in XBP1-overexpressed cells to determine whether the regulation is at the mRNA level.
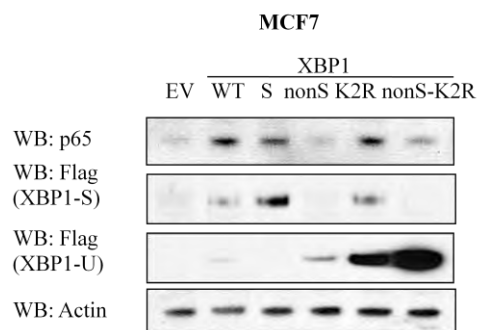
Figure 2. XBP1(S), not XBP(U), regulates RelA expression XBP1 or LacZ constructs were transfected into MCF7 cells, and harvest for western blots.
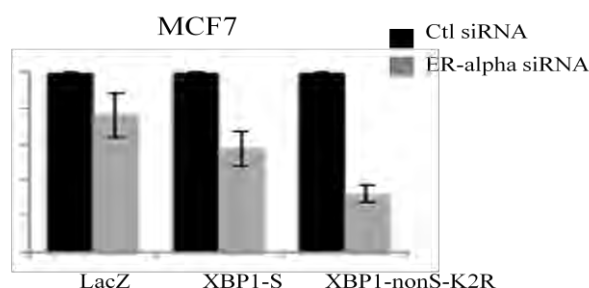

Figure 3. XBP1 regulation of NFkappaB transcriptional activity is ERalpha dependent. XBP1 or LacZ overexpressing MCF7 cells were co-transfected with ERalpha siRNA and NFkappaB luciferease construct. NFkappaB transcriptional activity was measured by luciferase assay.

XBP1 is known to interact with ERalpha and can regulate its transcriptional activity (7). As proposed in the plan, I next determined whether ERalpha is mediating the XBP1 regulation of RelA. To address this, we overexpressed XBP1 (U) and XBP1(S) in antiestrogen sensitive cells with or without ERalpha siRNA **(Figure 3)**. NFkappaB transcriptional activity is inhibited by ERalpha siRNA in XBP1(S) overexpressed cells, and further inhibition was observed in XBP1(U) overexpressed cells. However, we did not observe a difference in RelA expression, suggesting XBP1(S) regulation of RelA is independent of ERalpha signaling (**Figure 4**). Taking together, XBP1(S) regulates NFkappaB signaling mainly through RelA, whereas XBP1(U) regulates NFkappaB signaling mainly through ERalpha signaling (**Figure 5**).
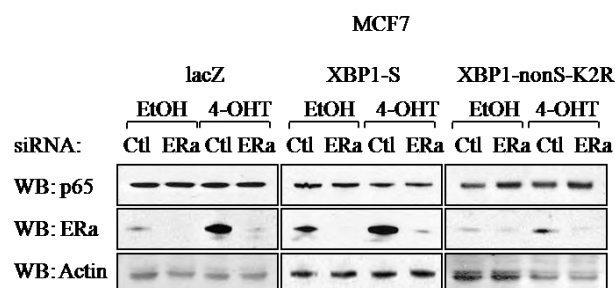

Figure 4. XBP1(S) regulation of RelA expression is independent of ERalpha signaling. XBP1 or LacZ overexpressing MCF7 cells were co-transfected with ERalpha siRNA.
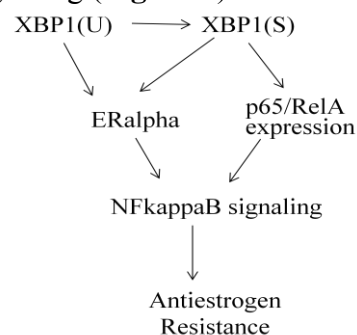

Figure 5. XBP1 regulates NFkappaB signaling through ERalpha-dependent and –independent signaling.

SubAim 3: Determine if NFkappaB signaling is required for XBP1-induced antiestrogen resistance.

To determine whether NFkappaB signaling is required for XBP1(S)-mediated antiestrogen resistance, we transfected p65 siRNA to inhibit NFkappaB signaling **(Figure 6)**. We found that inhibition of NFkappaB re-sensitizes XBP1 overexpressing cells to Tamoxifen. Furthermore, XBP1 overexpressing cells became strongly dependent on NFkappaB for survival. NFkappaB depletion alone induces apoptosis in XBP1 overxpressed cells, but not in lacZ control cells. Further apoptosis induction is observed when cells were co-treated with p65 siRNA and tamoxifen. These data suggest that NFkappaB signaling is required for XBP1-induced antiestrogen resistance.
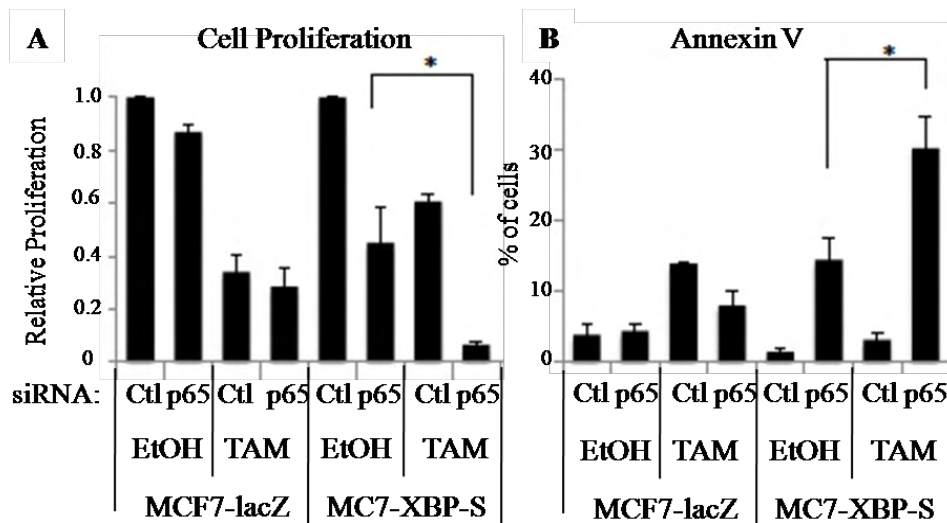
Figure 6. NFkappaB signaling is required for XBP1-mediated antiestrogen resistance. NFkappaB inhibition p65 knockdown inhibit cell growth (A) in XBP1-S overexpressing MCF7 cells, but not in LacZ cells. Same as (B) except apoptosis level are determined by Annexin V staining.

Aim 2: Determine the role of XBP1 in breast cancer antiestrogen resistance *in vivo.*
The first step for this aim is to generate MCF7 and LCC1 cell lines that will inducibly overexpress XBP1 in the presence of doxycycline. First, I infected MCF7 and LCC1 cells with TetR encoding lentivirus. After selected a single clone with high TetR expression level, I infected MCF7-TetR cells with XBP1 encoding lentivirus. Unfortunately, the cell lines I generated start to overexpress XBP1 constitutively, instead of in an inducible manner, after a few passages. We suspect that the growth advantage given by XBP1 overexpression promoted the loss of TetR repressor expression. Faced with this setback, I will re-establish the XBP1 inducibility in these cell lines by repetitively re-infecting these cells with TetR encoding lentivirus. Once I validate the inducible system, cells will be injected into nude mice as describe in the proposal, and doxycycline will be administered to the mice through drinking water to induce XBP1 expression.

Over the past year, I was successfully added to my mentor Dr. Robert Clarke's animal protocol, and the study I proposed was reviewed and approved by the IACUC in my host institution (GUACUC). After I obtain the final approval for NFkappaB inhibitor Parthenolide usage, I will submit my animal protocol to DOD-IACUC for review. Until I was granted with the final approval from DOD-IACUC, I was and will continue to prepare for the animal study that I proposed. I have been taking animal training in Georgetown University animal facility to learn to basic animal handling techniques. In addition, I will learn nude mice injection techniques with LCC1 and LCC9 cells from other lab members who are experts on animal work.

III. Key Research Accomplishments
- Depletion of XBP1 inhibits cell growth in antiestrogen resistant cells.
- Inhibition of XBP1 down-regulates NFkappaB signaling in antiestrogen resistant cells.
- XBP1(U) and XBP1(S) up-regulates NFkappB signaling through ERalpha.

- XBP1(S) regulates p65/RelA expression at the mRNA level, and this regulation is independent of ERalpha signaling.
- NFkappB signaling is required for XBP1 mediated antiestrogen resistance.

IV. Reportable Outcomes
Conference Abstracts:
1). "XBP1 regulates NFkB Signaling in antiestrogen resistant breast cancer cells"

Rong Hu, Ahreej Eltayeb, Ayesha Shajahan, Rebecca Riggins, Robert Clarke, Georgetown University Lombardi Comprehensive Cancer Center

Postser Presentation at the 2011 Annual Meeting of the AACR, April 2-6, in Orlando, FL

2)." NFkB Signaling is required for XBP1-mediated antiestrogen resistance in breast cancer"
Rong Hu, Ahreej Eltayeb, Ayesha Shajahan, Rebecca Riggins, Robert Clarke, Georgetown University Lombardi Comprehensive Cancer Center

Poster Presentation at the Era of Hope Meeting, August 2-5, in Orlando, FL

Manuscripts in preparation:
Hu R., Eltayeb A., Tabor K., Shajahan A., Riggins R., Clarke R. NF☐ B Signaling up-regulation is required for XBP1 Mediated Antiestrogen Resistance in Breast Cancer Cells. Manuscript in preparation.

V. Conclusions
From the first year of the funded research, we have made several positive findings. First, we confirmed that XBP1 is essential for antiestrogen resistance in breast cancer. Second, we found that XBP1 positively regulates NFkappaB signaling, partially through ERalpha signaling. Third, we showed that NFkappaB signaling is required for XBP1-mediated antiestrogen resistance.

Our current data established a link between XBP1 and NFkappaB signaling pathways. However, detailed mechanisms are still unclear and need to be further investigated. For example, even though we showed that XBP1(-S) regulates RelA mRNA in an ERalpha-independent manner, whether this regulation is achieved through direct binding of XBP1-S on RelA promoter or through a third player is not known. *In silico* analysis identified three potential binding sites for XBP1 on RelA promoter. In the follow-up study, I would like to determine whether XBP1 regulates RelA transcription as a direct transcription factor. I will perform ChIP analysis to test direct binding. I will also further determine the role apoptosis and autophagy played in XBP1-NFkappaB-mediated antiestrogen resistance.

As a survival signaling pathway that has been activated in many cancer types, NFkappaB signaling is an active target for therapeutics. According to the data I obtained from my first year's study, NFkappaB signaling plays an essential role in XBP1-driven antiestrogen resistance in breast cancer. Co-treatments that targeting both signaling pathways for synergistic effects should be examined for antiestrogen resistant breast cancer model. The animal studies as described in the proposal will serve to test this hypothesis.

VI. References

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2. Gomez, B.P., Riggins, R.B., Shajahan, A.N., Klimach, U., Wang, A., Crawford, A.C., Zhu, Y., Zwart,A., Wang, M., and Clarke, R., *Human X-box binding protein-1 confers both estrogen independence andantiestrogen resistance in breast cancer cell lines.* FASEB J., 2007. **21**(14): p. 4013-27.
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5. Riggins, R.B., Zwart, A., Nehra, R., and Clarke, R., *The nuclear factor kappa B inhibitor parthenolide restores ICI 182,780 (Faslodex; fulvestrant)-induced apoptosis in antiestrogen-resistant breast cancer cells.* Mol Cancer Ther, 2005. **4**(1): p. 33-41.
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VII. Appendices

1. Abstract presented at the 2011 Annual Meeting of the AACR (1 page).
2. Abstract presented at the 2011 Era of Hope meeting (1 page).
3. Current *curriculum vitae* (3 pages).

American Association for Cancer Research 102nd ANNUAL MEETING
April 2-6, 2011 • Orange County Convention Center, Orlando, Florida

Presentation Abstract

Abstract Number:	LB-148
Presentation Title:	XBP1 regulates NFkappaB signaling in antiestrogen resistant breast cancer cells
Location:	Exhibit Hall A4-C, Poster Section 39
Author Block:	Rong Hu, Ahreej Eltayeb, Ayesha Shajahan, Rebecca Riggins, Robert Clarke. Georgetown University Medical Center, Washington, DC
Abstract Body:	Unfolded protein response (UPR), a stress-induced survival mechanism, may be hijacked by cancer cells to avoid cell death. Therefore, the functional role of UPR during drug resistance has attracted an increasing amount of attention recently. Antiestrogen therapy, used in the treatment of estrogen receptor-positive (ER+) breast cancer, induces UPR. Upon endoplasmic reticulum stress, three arms of UPR signaling become activated to cope with stress conditions. One critical player that is regulated by two arms of the UPR signaling is a transcriptional factor called X-box binding protein 1 (XBP1). XBP1 exists in two forms, the transcriptionally inactive unspliced XBP1(U) and the spliced, actived XBP1(S). We have previously shown that overexpression of XBP1(S) confers estrogen independence and antiestrogen resistance in ER+ breast cancer cells. Others subsequently reported that XBP1(S) expression in ER+ breast tumors correlates with poor clinical responsiveness to Tamoxifen. However, the underlying signaling mechanisms affected by XBP1(S) as well as the effects of splicing on antiestrogen resistance remain unclear. We hypothesize that XBP1(S) mediates antiestrogen resistance in part through regulating nuclear factor kappa B (NFkappaB) signaling, since we previously showed that NFkappaB expression and activity are up-regulated in antiestrogen resistant cells, possibly through up-regulation of IKKgamma (NEMO) and RelA (NFkappaB p65). We now show that XBP1 regulates the expression of RelA in breast cancer cells. Overexpression of XBP1 in MCF7 and LCC1 antiestrogen sensitive breast cancer cells results primarily in an increase in XBP1(S) and an induction of RelA expression at both the mRNA and protein levels. We also show that NFkappaB transcriptional activity is upregulated by XBP1 overexpression. Moreover, the antiestrogen resistance conferred by XBP1 overexpression in MCF7 cells requires activated NFkappaB signaling. The presence of small molecule NFkappaB inhibitor, Parthenolide, sensitizes XBP1 ovexpressing MCF7 cells to Tamoxifen. Depletion of endogenous XBP1 using siRNA in antiestrogen resistant LCC9, LY2, and MCF7-RR breast cancer cells decreases RelA expression and NFkappaB transcriptional activity. Taken together, our findings establish a regulatory link between the UPR/XBP1 pathway and pro-survival NFkappaB signaling. Currently, we are actively investigating the role of XBP1 splicing in regulating NFkappaB signaling and antiestrogen resistance with a non-splicible mutant of XBP1 (XBP1(nonS)). In future studies, we will identify the pathways induced by XBP1/NFkappaB signals and further delineate their role in antiestrogen resistance in ER+ breast cancer.

American Association for Cancer Research
615 Chestnut St. 17th Floor
Philadelphia, PA 19106

Poster P41-23

BC100073-2811

XBP1 REGULATES NFkappaB SIGNALING IN ANTIESTROGEN-RESISTANT BREAST CANCER CELLS

Hu Rong, Ahreej Eltayeb, Ayesha Shajahan, Rebecca Riggins, and Robert Clarke
Georgetown University

The functional role of the unfolded protein response (UPR) during cancer progression has attracted an increasing amount of attention recently. As a stress-induced survival mechanism, UPR may be hijacked by cancer cells to avoid cell death. Upon endoplasmic reticulum stress, three arms of UPR signaling become activated to cope with stress conditions. Antiestrogen therapy, used in the treatment of estrogen receptor-positive (ER+) breast cancer, induces UPR. One critical player that is regulated by two arms of the UPR is the transcription factor X-box binding protein 1 (XBP1). XBP1 exists in two forms: the transcriptionally inactive unspliced XBP1(U) and the spliced, activated XBP1(S). We have previously shown that overexpression of XBP1(S) confers estrogen independence and antiestrogen resistance in ER+ breast cancer cells. Others subsequently reported that XBP1(S) expression in ER+ breast tumors correlates with poor clinical responsiveness to tamoxifen. However, the underlying signaling mechanisms affected by XBP1(S) that induce antiestrogen resistance remain unclear. We hypothesize that XBP1(S) mediates antiestrogen resistance in part through regulating nuclear factor kappa B (NFkappaB) signaling since we previously showed that NFkappaB expression and activity are upregulated in antiestrogen-resistant cells, possibly through upregulation of IKKgamma (NEMO) and RelA (NFkappaB p65). We now show that XBP1 regulates the expression of RelA in breast cancer cells. Overexpression of XBP1 in MCF7 and LCC1 antiestrogen-sensitive breast cancer cells results primarily in an increase in XBP1(S) and an induction of RelA expression at both the mRNA and protein levels. We also show that NFkappaB transcriptional activity is upregulated by XBP1 overexpression. Moreover, XBP1-overexpressing LCC1 cells show high dependency on NFkappaB signaling for proliferation as parthenolide, the small-molecule NFkappaB inhibitor, strongly inhibits cell growth in LCC1 cells overexpressing XBP1. Depletion of endogenous XBP1 using siRNA in antiestrogen-resistant LCC9, LY2, and MCF7-RR breast cancer cells decreases RelA expression and NFkappaB transcriptional activity. Taken together, our findings establish a regulatory link between the UPR/XBP1 pathway and pro-survival NFkappaB signaling. In future studies, we will identify the pathways induced by XBP1/NFkappaB signals and further delineate their roles in antiestrogen resistance in ER+ breast cancer.

BIOGRAPHICAL SKETCH

Provide the following information for the key personnel on page 1 of the Detailed Cost Estimate form for the initial budget period.

NAME	POSITION TITLE
Rong Hu	Post-doctoral Fellow

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, and include postdoctoral training.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	YEAR(s)	FIELD OF STUDY
Nanchang University	B.S.	1999-2003	Biotechnology
University of Leicester	M.Sc.	2003-2004	Molecular Genetics
SUNY-Albany		2004-2005	Molecular Biology
Albany Medical college		2005-2008	Cancer Biology
Thomas Jefferson University	Ph.D	2008-2009	Genetics
Georgetown University Medical Center	Post-Doc	2010-Present	Breast Cancer

RESEARCH AND PROFESSIONAL EXPERIENCE: Concluding with present position, list in chronological order, previous employment, experience, and honors. Include present membership on any Federal Government public advisory committee. List in chronological order the titles, all authors, and complete references to all publications during the past 3 years and to representative earlier publications pertinent to this application. If the list of publications in the last 3 years exceeds 2 pages, select the most pertinent publications. PAGE LIMITATIONS APPLY. DO NOT EXCEED 4 PAGES FOR THE ENTIRE BIOGRAPHICAL SKETCH PER INDIVIDUAL.

Research Experiences:

09/2003-08/2004:   MSc. In Molecular Genetics, University of Leicester, Leicester, UK
Thesis: Mechanism of Translational Selection of mRNAs during Apoptosis
Mentor: Dr. Martin Bushell

06/2005-11/2009:   Ph.D in Genetics, Albany Medical College and Thomas Jefferson University
Thesis: F-box proteins and co-factors of SCF E3 ubiquitin ligases in melanoma.
Mentor: Dr. Andrew Aplin

01/2010-Present: Post-doctoral Fellow, Georgetown University
Research: Role of UPR signaling in endocrine resistance in breast cancer
Mentor: Dr. Robert Clarke

Publications and Manuscript in preparation:

Bhatt KV., **Hu. R.,** Spofford LS., Aplin AE. (2007) Mutant B-RAF signaling and cyclin D1 regulate Cks1/S-phase kinase-associated protein 2-mediated degradation of p27Kip1 in human melanoma cells. Oncogene. 26(7): 1056-1066.

Hu R. and Aplin AE. (2008) Skp2 regulates G2/M progression in a p53-dependent manner. Mol. Biol. Cell. 19(11): 4620-4610.

Hu R. and Aplin AE. (2009) αB-crystallin is mutant B-RAF regulated and contributes to cyclin D1 turnover in melanocytic cells. Pigment Cell & Melanoma Research. 2010 Jan 27. Epub ahead of print.

Hu R., Eltayeb A., Tabor K., Shajahan A., Riggins R., Clarke R. NFkappaB Signaling up-regulation is required for XBP1 Mediated Antiestrogen Resistance in Breast Cancer Cells. Manuscript in preparation.

Funding:

Pre-doctoral Fellowship, National Cancer Center, 09/2007-09/2009
Skp2 regulation of melanoma cell proliferation: mechanism and role in a skin-like microenvironment

Post-doctoral Fellowship, Department of Defense, 09/2010-09/2013
Role of NFkB signaling in X-box binding protein 1(XBP1)-mediated antiestrogen resistance in breast cancer

Conferences:

Nov. 2007 International Melanoma Congress New York, NY
Poster: p53 and cyclin E1-dependent effects of Skp2 on melanoma cell cycle

Apr. 2008 AACR Annual Meeting San Diego, CA
Poster: Skp2 regulates G2/M progression in a p53-dependent manner

Sep. 2009 PanAmerican Society for Pigment Cell Research Annual Meeting Memphis, TN
Poster: F-box protein co-factor Cks1 and αB-crystallin: B-RAF regulation and roles in melanoma cell cycle progression

Apr. 2011 AACR Annual Meeting Orlando, FL
Poster: XBP1 regulates NFkB signaling in Antiestrogen resistant breast cancer cells

Apr. 2011 Experimental Biology Annual Meeting Washington, DC
Poster: IRF1 promotes antiestrogen sensitivity by regulating Bik expression in breast cancer cells

Aug. 2011 Era of Hope Breast Cancer Meeting Orlando, FL
Poster: NFkB Signaling is required for XBP1-mediated antiestrogen resistance in breast cancer

Awards and Honors:

Distinguished Student Award, Nanchang University, 2002, 2003

Student Scholarship, Nanchang University, 2000, 2001, 2002, 2003

Distinguished Student, University of Leicester, 2004

Richard A. Miller Alumni Prize, Albany Medical College, 2008

Dean's Excellence in Extramural Research Activities, Albany Medical College, 2008

Travel Award for 15th Annual Meeting of the PanAmerican Society for Pigment Cell Research, 2009

1st place in Poster Award for 15th Annual Meeting of the PanAmerican Society for Pigment Cell

Research, 2009

Mentoring:

Irene Thung: Irene was a medical student at Georgetown University who performed her research intern in Dr. Clarke's laboratory. In summer 2010, I worked with Irene toward the completion of her research project, which focuses on understanding the effects of Akt/mTOR dual inhibitors in antiestrogen-resistant breast cancer.

Ahreej Eltayeb: Ahreej was a graduate student at George Washington University who performed her research in Dr. Clarke's laboratory. From November 2010, I worked with Ahreej toward investigating the role of XBP1 splicing in antiestrogen resistance in breast cancer. I assisted Ahreej in crafting her research plan for the supplemental RO1 award that she received. Ahreej is now working as a full-time technician in Dr. Clarke's lab.

Katie Tabor: Katie is a medical student at Georgetown University working in Dr. Clarke's laboratory for her independent study project. From summer 2011, I worked with Katie on her project on understanding the role of unspliced form of XBP1 (XBP1-U) in apoptosis and autophagy in breast cancer.